SCIENTIFIC REPORT

Human papillomavirus and pterygium. Is the virus a risk factor?

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Background: Pterygium is a disease of unknown origin and pathogenesis that might be vision threatening. It is characterised by a wing-like conjunctival overgrowth of the cornea. Several studies have investigated human papillomavirus (HPV) as a risk factor for the development of pterygia, but the results are inconclusive.

Aim: To investigate a large sample of pterygia for the presence of HPV in order to clarify the putative association between pterygia and HPV.

Methods: 100 specimens of pterygium from Danish patients and 20 normal conjunctival biopsy specimens were investigated for the presence of HPV with PCR technique using β-globin primers to access the quality of the extracted DNA and the HPV primers MY09/11 and GP5+/6+. HPV-positive specimens underwent subsequent HPV typing with type-specific HPV primers and further investigation with DNA in situ hybridisation (ISH).

Results: 90 of 100 investigated pterygia proved suitable for HPV analysis by PCR. As β-globin could not be amplified, 10 specimens were excluded from the study. 4 of 90 pterygia harboured HPV. HPV type 6 was identified in all four HPV-positive pterygia. The 20 normal conjunctival biopsy specimens were β-globin positive and HPV negative. All four pterygia that were HPV type 6 positive were DNA ISH negative.

Conclusions: The low presence of HPV DNA in pterygia does not support the hypothesis that HPV is involved in the development of pterygia in Denmark.

Pterygium is a disease of unknown origin and pathogenesis that might be vision threatening. The disease is characterised by a wing-like conjunctival overgrowth of the cornea (fig 1).[1] The overgrowth consists of a stromal part containing fibroblasts and blood vessels and is accompanied by an inflammatory cell infiltrate and an abnormal accumulation of extracellular matrix, which is composed of elastin and collagen[1] (fig 1). The stromal part is covered by flattened conjunctival epithelium (fig 1). Bowman's layer beneath the pterygium may undergo destruction by the advancing fibrovascular tissue, resulting in a corneal scar. Pterygium is a common eye disease and has a worldwide distribution, occurring at highest prevalence and most severely in tropical regions. In Denmark, the prevalence of pterygium has been estimated to be 0.7%,[2] whereas the prevalence was found to be 9.9% in Singapore[3] and 23.4% among the coloured population of Barbados.[4] Originally believed to be a degenerative condition of the conjunctiva, recent theories are considering pterygium as a growth disorder.[5]

There is compelling epidemiological evidence that damage mediated by ultraviolet (UV) light acts as a trigger for the pathogenesis of pterygium.[3 4 6–9] In accordance with this, a "two-hit" theory has been suggested.[10] Detorakis *et al*[10] suggest that pterygium is caused due to a combination of an oncogenic agent and a pre-existing genetic damage produced by UV light (or inherited factors).

Human papillomavirus (HPV) is composed of a closed circular double-stranded DNA genome of which at least 100 different HPV genotypes are fully sequenced (http://www.stdgen.lanl.gov). The different HPV subtypes are associated with primarily benign or malignant epithelial tumours—for example, HPV 6 and 11 are associated with benign neoplasia and HPV 16 and 18 are closely linked with malignancy of the uterine cervical squamous epithelium.[11] HPV is also associated with epithelial tumours of the conjunctiva, including conjunctival papilloma[12] and conjunctival carcinoma.[13] Several studies have investigated HPV as a risk factor for the development of pterygium, but the outcome seems inconclusive as the proportion of HPV-positive pterygia ranges from 0% to 100%[14–17] and the materials investigated were limited.

The purpose of the present study was to investigate a large material of pterygium for the presence of HPV in order to clarify the putative association between pterygium and HPV.

MATERIALS AND METHODS

In all, 100 specimens of primary pterygium from Danish patients registered during the period 1994–2003 were collected from the files of the Eye Pathology Institute, University of Copenhagen, Copenhagen, Denmark. Gender and age of patients were registered, and all material were reviewed histologically for the confirmation of the diagnosis. Furthermore, 20 normal conjunctival biopsy specimens excised during the years 2000–1 were included as controls.

Polymerase chain reaction

Three sections from each paraffin wax block were cut. The sections were placed in 1.5 ml tubes and 50 µl digestion buffer (10 mM Tris pH 7.0, 1 mM EDTA and K 200 µg/ml proteinase) was added. The specimens were digested at 65℃ for 3 h, spinned and were transferred to a new tube for the aqueous phase. Boiling for 10 min inactivated the proteinase K. The samples, together with appropriate positive and negative controls, were amplified with primers targeting a 288 bp fragment of the single copy β-globin gene in order to ensure the integrity of the DNA as described by Sebbelov *et al*.[18] β-Globin-negative pterygia were excluded from further analysis.

HPV PCR was performed with the generally accepted consensus primers GP5+/GP6+[19] and MY 09/11[20]; these primers amplify the majority of the known mucosa-tropic HPV types. The PCR product, 15 µl, was run in a 1.5% submerged agarose gel in Tris-acetate-EDTA buffer, stained with ethidium bromide and viewed in UV transillumination. The sample was

Abbreviations: HPV, human papillomavirus; ISH, in situ hybridisation; UV, ultraviolet

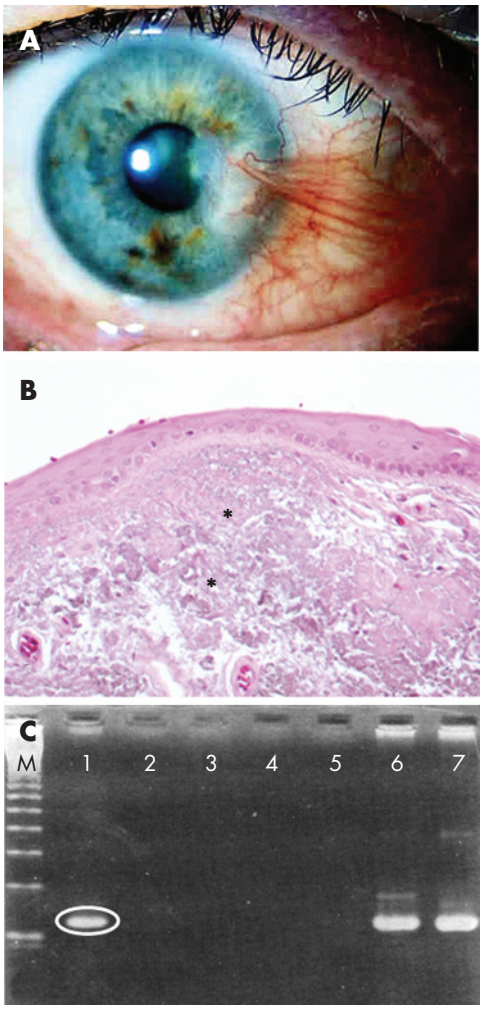

Figure 1 (A) Pterygium characterised by a wing-like conjunctival overgrowth of the cornea. (B) The basophilic appearance of elastotic degeneration is a characteristic feature of the stromal layer of pterygium. (H&E, ×100). (C) Agarose gel with PCR products viewed in ultraviolet-light transillumination. Lane M contains the DNA band marker that is used to estimate the size of the amplificated products. A circle marks the human papillomavirus (HPV) type 6 positive control in lane 1. Lanes 2 and 5 are negative controls, whereas lanes 3 and 4 are HPV-negative specimens. Lanes 6 and 7 are considered HPV type 6 positive due to the presence of a DNA band of the appropriate size.

considered HPV positive when a band of a predicted size (~150 bp for the GP5+/GP6+ primers or 450 bp for the MY09/11 primers) was identified on the gel. HPV-positive specimens underwent subsequent HPV typing with type-specific HPV primers 6, 11, 16, 18, 31 and 33.[21]

DNA in situ hybridisation

HPV PCR-positive pterygia and two HPV PCR-negative conjunctival biopsy specimens were investigated with DNA in situ hybridisation (ISH). Sections of 5 µm thickness were cut and then immersed successively in toluene, 100%, 90%, 60% and 30% ethanol, and distilled water. For unmasking of nucleic acids, the specimens were treated with 50 µg/ml of proteinase K for 20 min at 37°C. Denaturation of target DNA and probe was achieved by heat treatment at 75°C for 10 min and 95°C for 5 min, respectively. Specimens were incubated with 50 ng/µl digoxigenin-labelled HPV DNA probe 6, 11 or 16 at 37°C overnight. Sections were then subjected to two post-hybridisation washes in standard saline citrate buffer and incubated in phosphate-buffered saline containing 5% (weight/volume) bovine serum albumin. Thereafter, sections were incubated for 1 h with alkaline phosphatase conjugated anti-digoxigenin 1/2000, followed by precipitation with nitroblue tetrazolium chloride and bromochloroindoylphosphate. Finally, sections were counterstained for 10 s in Mayers haematoxylin. Positive control sections were included in the experiment.

RESULTS

Patient characteristics

The 100 patients consisted of 57 men and 43 women. Age range was 37–85 years, with a mean age of 63.4 years. All pterygia were primary lesions.

PCR results

In all, 90 of 100 investigated pterygia proved suitable for HPV analysis by PCR. As β-globin could not be amplified in 10 specimens these were excluded from the study. In all, 4 of 90 pterygia harboured HPV. HPV type 6 was identified in all four HPV-positive pterygia (fig 1). All 20 normal conjunctival biopsy specimens were β-globin positive and HPV negative.

DNA ISH results

The four pterygia that were HPV type 6 positive had been analysed using PCR investigations, and all were DNA ISH negative.

DISCUSSION

UV light is associated with the development of pterygium,[8] which is observed only in humans, and the lesion has never been described in animals. Therefore, risk factors other than UV radiation must be significant in the pathogenesis of pterygium. Our investigation of 100 pterygia questions the possibility that HPV is involved in the pathogenesis of pterygium. The low-risk HPV type 6 was identified in only four specimens using the PCR technique. However, subsequent DNA ISH investigations were unable to confirm the presence of HPV. It is well known that the PCR technique is superior in sensitivity to the ISH technique.[18 22] Therefore, the four HPV PCR-positive but HPV DNA ISH-negative specimens could be due to contamination of the investigated specimens or a non-significant, incidental occurrence of an actual infection with a low number of virions. Nonetheless, owing to the small number of HPV-positive pterygia in our material, it seems unlikely that HPV is an essential factor in the development of pterygia in Denmark.

Other investigations of pterygium have shown great geographical variance in the proportion of HPV-positive pterygium.[14–16 23 24] Using PCR, HPV was not identified in 65 pterygia from Taiwan[14] and, likewise, Dushku *et al*[24] were unable to detect HPV in an investigation of 13 pterygia in a study from the USA. On the other hand, 30% of pterygium from Greek patients were HPV positive[23]; in a British study,[15] the HPV prevalence was 50% and Piras *et al*[16] found HPV in 100% of 17 investigated Italian pterygia. However, none of the previous investigations have used immunohistochemistry or ISH to show the localisation of HPV in their HPV-positive pterygia.[15 16 23 24]

A mechanism that might cause HPV to become undetectable by standard PCR assays is the integration of HPV DNA in the host cell genome in HPV-positive tissue. Integration of HPV results in the interruption of HPV DNA in the early region of the genome.[25] Nevertheless, previous studies performed on cervical carcinoma in The Netherlands indicate that this mechanism plays only a minor role.[26] The pathogenesis of HPV infections might also support that HPV is not associated with pterygium. HPV infections are confined to the keratinocytes of the skin and mucosal surfaces.[27] Therefore, the virus is highly adapted to this cell type and productive HPV infection is unlikely to occur in

any other tissue types.[27] Additionally, epidemiological data of pterygium prevalence and HPV infection contradict. The prevalence of pterygium increases linearly with age,[3 4] whereas HPV infection is predominantly seen among younger people in their 20s and 30s.[28] However, in recent years, new types of skin HPV have been identified and are more common than considered previously. These observations indicate that there is a reservoir of superficially located virus on the skin.[29] Perhaps the different HPV frequencies in geographically distinct populations reflect that pterygium is a multifactorial disease resulting from a non-specific response to various different insults. A number of proinflammatory cytokines, angiogenic and fibrogenic growth factors and their receptors have been reported in pterygium, which parallels their involvement in the corneal wound-healing cascade.[30 31] In particular, basic-fibroblast growth factor, matrix metalloproteinases, vascular endothelial growth factor and heparin-binding epidermal growth factor are over-expressed in pterygium and, furthermore, directly or indirectly, induced by UV light.[5 31] It has recently been shown that key proinflammatory cytokines and growth factors are modulated by exposure to UV light through intracellular pathways, which supports the role of cumulative UV damage in pterygium formation.[31] Other postulated causative agents of this process include herpes virus[23] and factors that cause repeated insults to the interpalpebral corneoscleral junction (eg, dust and wind exposure).[1]

In conclusion, our finding of the very limited presence of HPV DNA in pterygium does not support the hypothesis that HPV is involved in the development of pterygium in Denmark. The geographical variance in the proportion of HPV-positive pterygium may reflect that pterygium is a multifactorial disease.

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